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Glucose degradation product methylglyoxal enhances the production of vascular endothelial growth factor in peritoneal cells: role in the functional and morphological alterations of peritoneal membranes in peritoneal dialysis

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Abstract Peritoneal membrane permeability deteriorates in peritoneal dialysis (PD) patients. We test whether glucose degradation products (GDPs) in PD fluids, glyoxal, methylglyoxal and 3-deoxyglucosone, stimulate the production of vascular endothelial growth factor (VEGF), a factor known to enhance vascular permeability and angiogenesis. VEGF increased in cultured rat mesothelial and human endothelial cells exposed to methylglyoxal, but not to glyoxal or 3-deoxyglucosone. VEGF also increased in peritoneal tissue of rats given intraperitoneally methylglyoxal. VEGF and carboxymethyllysine (CML) (formed from GDPs) co-localized immunohistochemically in mesothelial layer and vascular walls of the peritoneal membrane of patients given chronic PD. By contrast, in the peritoneum of non-uremic subjects, VEGF was identified only in vascular walls, in the absence of CML. VEGF production induced by GDPs may play a role in the progressive deterioration of the peritoneal membrane.

Key words: Peritoneal dialysis; Glucose degradation product; Methylglyoxal; Vascular endothelial growth factor; Peritoneal membrane

1. Introduction

The quality of the peritoneal membrane deteriorates progressively with peritoneal dialysis (PD) duration [1]. From a morphological point of view, membrane alteration is characterized by interstitial fibrosis, disappearance of mesothelial cells, vascular wall thickening, vasodilation and increased angiogenesis [1,2]. From a functional point of view, ultrafiltration decreases with an eventual impairment of macromolecular diffusion in a few patients [3].

The glucose content of the dialysate has been incriminated in the alteration of the peritoneal membrane. Heat sterilization of PD fluids degrades glucose into a variety of carbonyl compounds such as methylglyoxal, glyoxal and 3-deoxyglucosone [4,5], all of which are cytotoxic and mitogenic [6,7]. These products also lead to advanced glycation of proteins [8,9], associated with age-related disorders and with diabetic or uremic complications [10–13]. The effect of advanced glycation end products (AGEs) on the peritoneal membrane remains to be elucidated.

Decreased ultrafiltration has been taken as evidence for an augmentation of the peritoneal surface area available for diffusive exchange [1]. An increased vascular surface area within the peritoneal membrane might thus account for the loss of ultrafiltration. Within this framework, the vascular endothelial growth factor (VEGF) might play a critical role. VEGF increases vascular permeability [14,15], stimulates nitric oxide synthase production and thus vasodilation [16], and initiates inflammatory responses [17,18]. Finally, it is a powerful angiogenic factor contributing to the development of vascular lesions [19–21].

The present paper assesses the possible role of VEGF in the modification of peritoneal membrane characteristics. More specifically, we examine the effect of methylglyoxal, a major glucose degradation product (GDP) [4,5], on the production of VEGF, both in vitro in cultured peritoneal mesothelial and endothelial cells and in vivo in peritoneal tissues of rats. We also examine by immunohistochemistry the distributions of VEGF and GDP-modified proteins in peritoneal tissues obtained from long-term PD patients. We report for the first time that methylglyoxal enhances the production of VEGF in peritoneal cells and propose the hypothesis that GDPs contained in PD fluids impair the permeability of the peritoneal membrane, at least in part, through an augmented production of VEGF and its attendant stimulation of angiogenesis.

2. Materials and methods

2.1. In vitro mesothelial and endothelial cell culture experiments

Peritoneal tissue was obtained from 6 week old male CD (SD) IGS rats (Charles-River, Kanagawa, Japan). Mesothelial cells were isolated according to the method of Hjelle et al. [22] and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Human microvascular endothelial cells were purchased from Kurabo (Osaka, Japan) and cultured in VEGF-depleted EGM-2 medium (Takara, Tokyo, Japan). The mesothelial and endothelial cells at passage 7–10 were cultured for 3 h in a CO_2 incubator in the presence of several concentrations of various GDPs, i.e. glyoxal, methylglyoxal (Sigma, St. Louis, MO, USA) or 3-deoxyglucosone (kindly provided

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Abbreviations: AGE, advanced glycation end product; PD, peritoneal dialysis; RT-PCR, reverse transcriptase-polymerase chain reaction; VEGF, vascular endothelial growth factor

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from Fuji Memorial Research Institute, Otsuka Pharmaceutical, Kyoto, Japan). Cellular VEGF mRNA expression was then evaluated as described below. Mesothelial cells were also cultured for 24 h in the presence of methylglyoxal and VEGF protein production was measured in the supernatant. The cell viability was tested by trypan blue exclusion.

2.2. In vivo animal experiments

Six week old male CD (SD) IGS rats were given a daily intraperitoneal injection of 50 ml/kg of a saline solution containing various concentrations of methylglyoxal for 10 days. Peritoneal membrane was obtained from the parietal walls and investigated for VEGF mRNA expression. All studies were conducted in accord with the NIH Guide for the Care and Use of Laboratory Animals. Protocols were approved by the Institutional Animal Care and Use Subcommittee.

2.3. Detection of VEGF expression

The expression of VEGF mRNA was analyzed by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated from mesothelial and endothelial cells using Rneasy Mini kit (Qiagen, Germany) and from peritoneal tissue by using ISOGEN (Nippon Gene, Tokyo, Japan). Five μg of the RNA was reverse-transcribed using oligo$(dT)_{12-18}$ primers (Gibco BRL, Gaithersburg, MD, USA) with 200 U of RNase H-free RT (Superscript II, Gibco BRL) and PCR amplification was performed as described previously [11]. The oligonucleotide primer sequences are as follows. Primers for rat VEGF were 5′-ACTGGACCCTGGCTTTACTGC-3′ and 5′-TTGGTGAGGTTTGATCCGCATG-3′: the full-length amplified fragment is 310 bp long. Primers for human VEGF were 5′-GGCAGAATCATCACGAAGTGGTG-3′ and 5′-CTGTAGGAAGCTCATCTCTCC-3′: the full-length amplified fragment is 271 bp long. The primers for rat and human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were 5′-CCTGCACCACCAACTGCTTAGCCC-3′ and 5′-GATGTCATCATATTTGGCAGGTT-3′ and amplified a 322 bp fragment. The G3PDH served as an internal RNA control to allow comparison of RNA levels among different specimens. Specimens were amplified in a DNA Thermal cycler (Perkin-Elmer Cetus, Norwork, CT, USA) for suitable cycles consisting of 0.5 min at 94°C, 1 min at 60°C and 1.5 min at 72°C. In preliminary experiments, reverse transcription and PCR amplification were performed on various amounts of RNA for 16, 18, 21, 25, 28, 31 and 34 cycles. These experiments showed that with 28 or 30 cycles of amplification for VEGF mRNA and with 16 or 21 cycles of G3PDH mRNA amplification, the differences in PCR product signal were quantitatively related to input RNA. PCR products resolved by electrophoresis in 1.5% agarose and stained by ethidium bromide were quantified by measuring the signal intensity with a quantitation program (NIH image). Experiments were performed for each GDP concentration. Messenger RNA was determined in triplicate and the results were averaged for each experiment. A total of 3–4 independent experiments were performed for each experimental condition. The results were averaged and expressed as mean ± S.D.

In some experiments, the VEGF protein was quantified in duplicate in the culture supernatant by enzyme-linked immunosorbent assay (ELISA) using a kit (Quantikine: R&D Systems, Minneapolis, MN, USA) according to the manufacturer's technical guidelines. The experiment was repeated three times.

2.4. Immunohistochemistry

Peritoneal tissues were obtained, with informed consent, from nine non-diabetic PD patients (Table 1) during catheter reinsertion. Reinsertion was necessitated by catheter failure due to damage, incorrect position and/or obstruction. The patients had never suffered from peritonitis. Normal peritoneal tissue was obtained, during abdominal surgery, from two male subjects (48 and 58 years old) with normal renal function. The study was approved by the Human Research Committee of Tokai University School of Medicine.

Two μm thick peritoneal tissue sections were mounted on slides coated with 3-aminopropyltriethoxy silane (Sigma, St. Louis, MO, USA), deparaffined, rehydrated in distilled water and incubated with pronase (0.5 mg/ml: Dako, Glostrup, Denmark) for 15 min at room temperature in a buffer solution containing 0.05 M Tris–HCl (pH 7.2), 0.1 M NaCl. The slides were washed with PBS containing 0.5% Tween 20, blocked in 4% skim milk for 2 h and subsequently incubated with anti-VEGF rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-AGE mouse IgG [23], the epitope structure of which was identified as carboxymethyllysine (CML) [24], overnight in humid chambers at 4°C. The sections were washed and incubated with 1:100 diluted goat anti-rabbit IgG conjugated with peroxidase or goat anti-mouse IgG conjugated with peroxidase (Dako, Glostrup, Denmark) for 2 h at room temperature, followed by the detection with 3,3′-diaminobenzidine solution containing 0.003% H_2O_2. Periodic acid-Schiff staining was also performed for histological study. Immunostaining was independently evaluated for intensity and distribution by two observers.

2.5. Statistical analyses

Data are expressed by mean ± S.D. Analysis of variance (ANOVA) was utilized to evaluate the statistical significance of various differences. If a significant difference was indicated by the analysis, Scheffe's *t*-test was used to compare results obtained with different concentrations of methylglyoxal.

3. Results

3.1. VEGF expression in mesothelial and endothelial cells cultured in the presence of GDPs

VEGF mRNA expression was assessed in mesothelial cells cultured in the presence of glyoxal, methylglyoxal or 3-deoxyglucosone. Despite concentrations varying from 0 to 400 μM, neither glyoxal nor 3-deoxyglucosone modified VEGF expression (data not shown). Only methylglyoxal stimulated VEGF mRNA expression at a concentration of 400 μM ($P < 0.0005$) (Fig. 1A). The sample RNAs that had not been reverse-transcribed did not yield the PCR product (lane 13 in Fig. 1). All cells remained viable. Mesothelial cells were also cultured in the presence of higher concentrations of 3-deoxy-

Table 1
Immunohistochemical detection of CML and VEGF in peritoneal tissues of PD patients

Samples	Gender	Age (years)	PD duration (months)	CML		VEGF	
				Mesothelial layer	Vascular walls	Mesothelial layer	Vascular walls
normal1	M	48	–	−	±	−	+
normal2	M	58	–	−	±	−	+
PD1	F	53	3	+	+	+	+
PD2	M	44	4	+	+	+	+
PD3	M	43	45	+	+	+	+
PD4	F	54	60	++	++	++	+
PD5	M	52	70	++	++	+	+
PD6	M	51	90	++	++	++	++
PD7	M	45	105	++	++	++	++
PD8	M	62	108	++	++	++	++
PD9	M	66	110	++	++	++	++

−: negative, ±: faint, +: positive, ++: strongly positive.

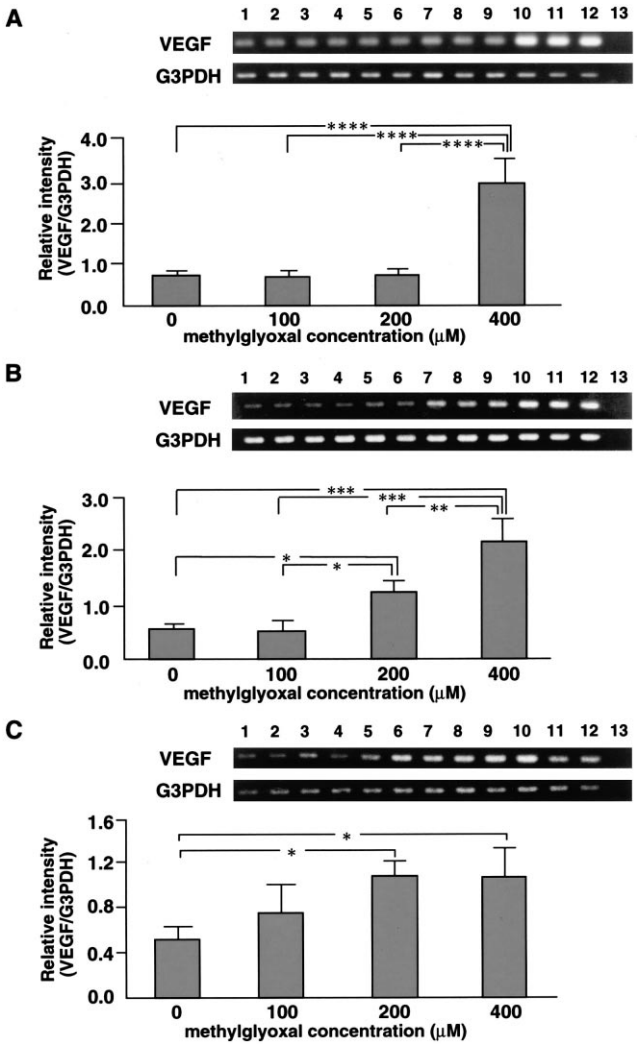

Fig. 1. VEGF mRNA expression in mesothelial cells, endothelial cells and peritoneal exposed to methylglyoxal. Reverse transcription was performed on total RNA from cultured rat mesothelial (A) and human endothelial cells (B) incubated with various concentrations of methylglyoxal and peritoneal tissues (C) of rats given daily intraperitoneal loads of methylglyoxal for 10 days. VEGF and G3PDH cDNAs from mesothelial and endothelial cells were then amplified by PCR for 30 and 21 cycles, respectively, and those from peritoneal tissues were amplified by PCR for 28 and 16 cycles, respectively. Data from one representative experiment performed in triplicate are illustrated in the top part of A–C. Lanes 1–3, total RNAs from the cells incubated with medium alone; lanes 4–6, 7–9 and 10–12, total RNAs from the cells incubated with 100, 200 and 400 μM methylglyoxal, respectively; lane 13, total RNAs from the cells incubated with 400 μM methylglyoxal without reverse transcription. Mean ratio of VEGF mRNA over G3PDH was calculated for each experiment. The average of the three experiments is expressed in the bottom part of A–C. $^{*}P<0.05$, $^{**}P<0.005$, $^{***}P<0.0001$, $^{****}P<0.0005$.

glucosone (0.625, 2.5 and 5 mM). Cells incubated with these high levels of 3-deoxyglucosone had a decreased viability (cell viability was 80, 55, 8% for 0.625, 2.5, 5 mM 3-deoxyglucosone, respectively). VEGF mRNA expression could therefore not be measured. As a consequence of these observations, only methylglyoxal was used in the subsequent experiments.

The release of VEGF protein in the supernatant was measured by ELISA after a 24 h culture of mesothelial cells in the presence of various concentrations of methylglyoxal. Addition of methylglyoxal to the medium resulted in a dose-dependent increase of VEGF (VEGF production was 22.57 ± 1.15, 29.24 ± 2.05, 37.85 ± 8.06 pg/ml for 0, 200, 400 μM, respectively). No VEGF release was detected during incubation without mesothelial cells (data not shown).

VEGF mRNA expression was then assessed in endothelial cells cultured in the presence of various concentrations (0–400 μM) of methylglyoxal. VEGF mRNA expression rose in a dose-dependent manner, as shown in Fig. 1B.

3.2. *VEGF expression in peritoneal tissues of rats given intraperitoneal injection of methylglyoxal*

The biological effects of methylglyoxal on VEGF mRNA expression in the peritoneum were further assessed in vivo in rats given various amounts of methylglyoxal for 10 days into the peritoneal cavity. As shown in Fig. 1C, VEGF mRNA expression in samples of the parietal peritoneum increased significantly ($P<0.05$) as a function of methylglyoxal concentration. On optic microscopy, the peritoneal tissue was unaffected: the number of vessels, the vascular wall, the interstitium and mesothelial cells remained normal.

3.3. *VEGF and CML immunostaining of peritoneal tissue of long-term PD patients*

The distribution of VEGF and CML was examined by immunohistochemistry in the peritoneal tissues of nine patients undergoing PD. CML is derived from GDPs such as glyoxal and 3-deoxyglucosone [9]. An anti-CML antibody was therefore used as a marker of GDP-modified proteins. Results are summarized in Table 1. Fig. 2A,D shows pictures of the peritoneal tissue of a representative long-term PD patient (PD6 in Table 1). It is characterized by interstitial fibrosis and thickening and hyalinosis of vascular walls. Both VEGF and CML co-localized in the mesothelial layers and in the vascular walls (Fig. 2B–F). Of note, in mesothelial layer, the signal with VEGF was weaker than that with CML. Results were similar in the eight other patients. Fig. 2G illustrates a normal peritoneal sample (normal2 in Table 1). In contrast with PD samples, VEGF was present only in the vascular walls (Fig. 2H) but was absent in the mesothelial layer (data not shown). CML was absent in the mesothelial layer (data not shown) and was very weak in the vascular walls (Fig. 2I). Observations were similar in the other control normal sample. No immunostaining was observed when normal mouse IgG was used (data not shown).

4. Discussion

The molecular events implicated in the dialysis-induced alteration of the peritoneal membrane are as yet unknown. We demonstrate for the first time a link between methylglyoxal, a GDP present in heat-sterilized PD fluids, and the stimulation of VEGF production by peritoneal mesothelial and endothelial cells.

Both levels of VEGF mRNA and protein production were significantly augmented by methylglyoxal in cultured mesothelial cells. No such phenomenon is observed in the presence of similar concentration of glyoxal or 3-deoxyglucosone. When the 3-deoxyglucosone concentration is raised to higher levels, cell viability decreases, thus preventing assessment of VEGF mRNA expression. The effect of methylglyoxal is not re-

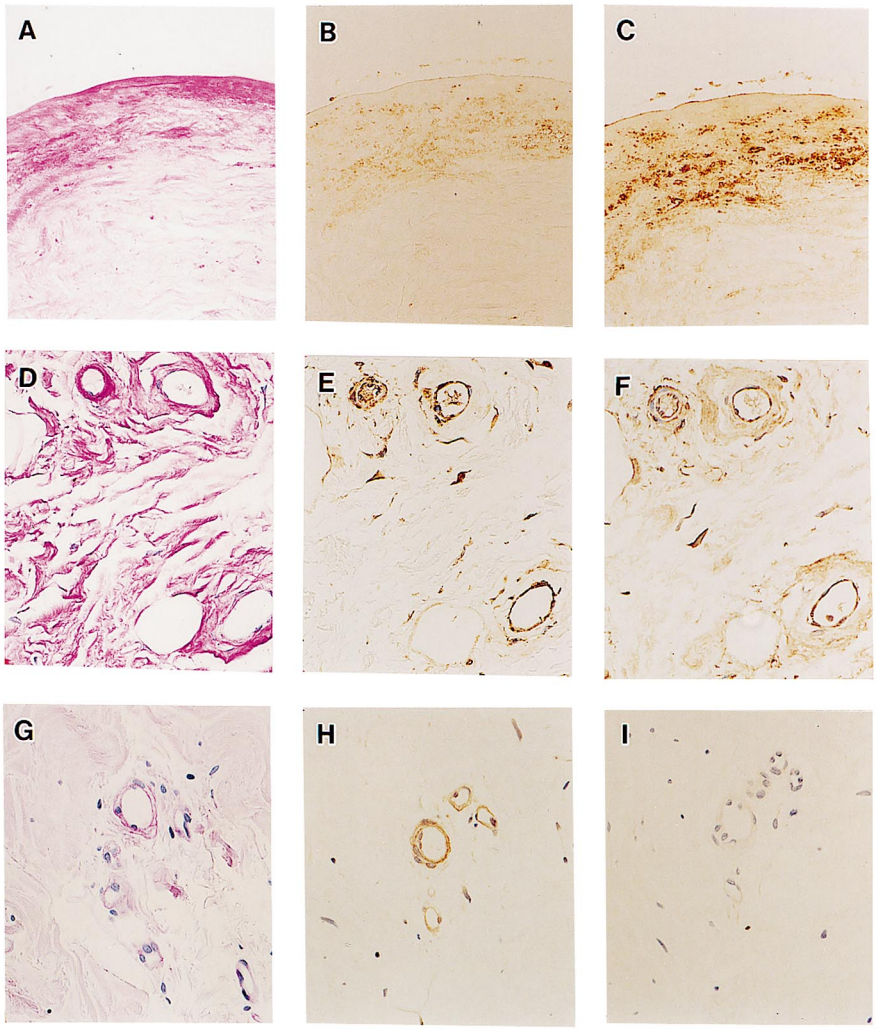

Fig. 2. Immunohistochemical detection of VEGF and GDP-modified proteins in peritoneal tissues from a long-term PD patient (PD6) and a normal subject (normal2). Peritoneal tissue sections from a 51 year old patient on PD for 90 months (A–F) and from a 58 year old non-uremic patients (G–I) were stained with Periodic acid-Schiff (left panels) or immunostained for VEGF (middle panels) and CML (right panels). VEGF and CML are both present in the mesothelial layer and in the vascular walls in the peritoneum of the long-term PD patient. By contrast, only VEGF is visible in the vessels of the peritoneum of the control subject. Nuclei were counterstained with Meyer's hematoxylin. Magnification, ×80.

stricted to mesothelial cells. It is also observed in cultured endothelial cells and in vivo in the peritoneum of rats given intraperitoneal injections of methylglyoxal.

The methylglyoxal level needed to enhance VEGF expression and release (∼400 μM) is 20–200 times above that observed in heat-sterilized PD fluid (2–23 μM) [4]. Appreciation of the clinical relevance of the methylglyoxal effect should take into account that, in vivo in man, duration of exposure of the peritoneal cells to the compound is much longer (several years) than in our experimental setting (3 h for in vitro cell culture and 10 days for in vivo rat study). The difference between methylglyoxal levels observed in PD fluid and that needed in vitro to stimulate VEGF synthesis further suggests that other compounds may have a similar effect. Such compounds need not to be derived from glucose degradation in PD fluid. They might also originate from the uremic serum and diffuse within the peritoneal fluid. We have recently demonstrated that such a mechanism accounted for the accumulation of pentosidine precursors in peritoneal dialysate during dwell [25,26].

Our immunohistochemical observations in human peritoneal tissue also support the existence of a link between VEGF synthesis and GDPs. In the absence of a specific antibody against methylglyoxal-modified proteins, we assumed that the factors governing AGE protein modification were similar for the various GDPs. We thus relied, as a substitute, on an antibody against CML, a protein modification induced by glyoxal and 3-deoxyglucosone. VEGF and CML indeed co-localize both in the mesothelial layer and in the vascular walls of peritoneal tissue obtained from PD patients. By contrast, in peritoneal samples of non-uremic subjects, VEGF was detected only in the vascular walls, in the absence of CML. Immunohistochemistry does not allow proper quantification of tissue VEGF. Nevertheless, the fact that VEGF extended to the mesothelial layers and co-localized with CML only in PD patients suggests that the GDPs present in PD fluid enhanced VEGF production in uremic patients given PD.

Increased VEGF production in peritoneal cells might account, in part, for the functional deterioration of the perito-

neal membrane. Ultrafiltration failure is its earliest and most frequent manifestation [3,27,28]. It results from an increased diffusion of glucose out of the peritoneal cavity, leading to early dissipation of the transmembrane osmotic gradient. This abnormality has been ascribed to an augmented vascular peritoneal surface area, consistent with recent evidence of an increased vascular density in peritoneal tissue of PD patients [1,29]. VEGF might augment the vascular peritoneal surface area by stimulating vascular permeability [14,15], vasodilation [16] and angiogenesis [19–21]. Further studies will undoubtedly unravel the effects of VEGF on the peritoneal membrane and clarify the role of nitric oxide release [2] as well as the consequences of the AGE transformation of peritoneal membrane proteins.

Methylglyoxal, just as other GDPs, such as glyoxal and 3-deoxyglucosone, contains two carbonyl groups able to cross-link cell surface proteins. We recently demonstrated that, in cultured rat sensory neurons, they thus led to the formation of AGE protein adducts [30]. In murine thymocytes and fibroblasts, the same phenomenon activates protein kinases such as c-Src and increases intracellular tyrosine phosphorylation of several cellular proteins [31]. We further suggested that the formation of carbonyl-mediated Schiff base with cell surface protein is crucial for generation of the signal, because 2-isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide (OPB-9195), a potent Schiff base inhibitor [32,33], inhibited the intracellular protein-tyrosine phosphorylation induced by glyoxal [31].

Much effort has been devoted to prevent the deterioration of the peritoneal membrane. The present study offers a rationale for reducing GDPs such as methylglyoxal by a filter sterilization of PD fluid or by the replacement of glucose by glucose polymers [1,34,35]. It should be emphasized first that methylglyoxal is probably only one of the compounds able to raise VEGF production and second, that enhanced VEGF synthesis may be one among several mediators of the functional deterioration of the peritoneal membrane.

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